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(71) Applicant *(for all designated States except US)*: UNIVERSITY OF SOUTHERN CALIFORNIA [US/US]; University Park, Los Angeles, CA 90089 (US).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: HORWITZ, David, A. [US/US]; University of Southern California, 2011 Zonal Avenue, HRM 711, Los Angeles, CA 90033 (US).

(74) Agents: TRECARTIN, Richard, F. et al.; Flehr, Hohbach, Test, Albritton & Herbert LLP, Suite 3400, 4 Embarcadero Center, San Francisco, CA 94111-4187 (US).

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(54) Title: USE OF CYTOKINES AND MITOGENS TO INHIBIT PATHOLOGICAL IMMUNE RESPONSES

(57) Abstract

Method for ex-vivo inhibition of the production of immunoglobulins in a sample of peripheral blood mononuclear cells (PBMC) by addition of an inhibitory composition (e.g. containing IL-2 or TGF-beta as inhibitor agent) to the cells. Method of treating autoimmune diseases, e.g. systemic lupus erythematosus (SLE), by reintroducing the treated cells to a patient.

USE OF CYTOKINES AND MITOGENS TO INHIBIT PATHOLOGICAL IMMUNE RESPONSES

FIELD OF THE INVENTION

The field of the invention is generally related to methods of treating antibody-mediated autoimmune diseases.

BACKGROUND OF THE INVENTION

Autoimmune diseases are caused by the failure of the immune system to distinguish self from non-self. In these diseases, the immune system reacts against self tissues and this response ultimately causes inflammation and tissue injury. Autoimmune diseases can be classified into two basic categories: antibody-mediated diseases such as systemic lupus erythematosus (SLE), pemphigus vulgaris, myasthenia gravis, hemolytic anemia, thrombocytopenia purpura, Grave's disease, Sjogren's disease and dermatomyositis; and cell-mediated diseases such as Hashimoto's disease, polymyositis, disease inflammatory bowel disease, multiple sclerosis, diabetes mellitus, rheumatoid arthritis, and scleroderma.

In many autoimmune diseases, tissue injury is caused by the production of antibodies to native tissue. These antibodies are called autoantibodies, in that they are produced by a mammal and have binding sites to the mammals own tissue. Some of these disorders have characteristic waxing and waning of the amount of autobodies circulating causing varying symptoms over time.

Of the different types of antibody-mediated autoimmune disorders, SLE is a disorder that has been well studied and documented. SLE is a disorder of generalized

and Wilkins, Baltimore). Although it is well recognized that the primary role of certain lymphocytes is to down-regulate immune responses, progress in elucidating the identity and mechanisms required for generation of these cells has been slow.

Interleukin-2 (IL-2) has previously been considered to have an important role in the generation of antigen non-specific T suppressor cells. Anti-IL-2 antibodies given to mice coincident with the induction of graft-versus-host-disease resulted in several features of SLE (Via, C.S. *et al.* (1993), *International Immunol. 5:*565-572). Whether IL-2 directly or indirectly is important in the generation of suppression has been controversial (Fast, L.D. (1992), *J. Immunol. 149:*1510-1515; Hirohata, S. *et al.* (1989), *J. Immunol. 142:*3104-3112; Baylor, C.E. (1992), *Advances Exp. Med. Biol. 319:*125-135). Recently, IL-2 has been shown to induce CD8+ cells to suppress HIV replication in CD4+ T cells by a non-lytic mechanism. This effect is cytokine mediated, but the specific cytokine has not been identified (Kinter, A.L. *et al. Proc. Natl. Acad. Sci. USA 92:*10985-10989; Barker, T.D. *et al.* (1996), *J. Immunol. 156:*4478-4483). T cell production of IL-2 is decreased in SLE (Horwitz, D.A. *et al.* (1997), *Dubois' Lupus Erythematosus*, 5th Ed. (1997), pp. 83-96, D.J. Wallace *et al.* eds., Williams and Wilkins, Baltimore).

CD8+ T cells from subjects with SLE sustain rather than suppress polyclonal IgG production (Linker-Israeli, M. *et al.* (1990), *Arthritis Rheum. 33:*1216-1225). CD8+ T cells from healthy donors can be stimulated to enhance Ig production (Takahashi, T. *et al.* (1991), *Clin. Immunol. Immunopath. 58:*352-365). However, neither IL-2 nor CD4+ T cells, by themselves, were found to induce CD8+ T cells to develop strong suppressive activity. When NK cells were included in the cultures, strong suppressive activity appeared (Gray, J.D. *et al.* (1994) *J. Exp. Med. 180:*1937-1942). It is believed that the contribution of NK cells in the culture was to produce transforming growth factor beta (TGFβ) in its active form. It was then discovered that non-immunosuppressive (2-10 pg/ml) concentrations of this cytokine served as a co-factor for the generation of strong suppressive effects on IgG and IgM production (Gray, J.D. *et al.* (1994) *J. Exp. Med. 180:*1937-1942). In addition, it is believed that NK cells are the principal source of TGFβ in unstimulated lymphocytes (Gray, J.D. *et al.* (1998), *J. Immunol. 160:*2248-2254).

TGFβ is a multifunctional family of cytokines important in tissue repair, inflammation and immunoregulation (Massague, J. (1980), *Ann. Rev. Cell Biol. 6:*597). TGFβ is unlike most other cytokines in that the protein released is biologically inactive and unable to bind to specific receptors (Sporn, M.B. *et al.* (1987) *J. Cell Biol. 105:*1039-

In an additional aspect, the invention provides kits for the treatment of an autoimmune disorder in a patient. The kits comprise a cell treatment container adapted to receive cells from a patient with an antibody-mediated autoimmune disorder and at least one dose of an inhibitory composition.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows that incubation of SLE patients PBMC with IL-2 and TGF-β decreases spontaneous immunoglobulin production. PBMC ($2x10^5$/well) were cultured in AIM-V serum free medium with or without IL-2 (10U/ml) and TGF-β (10pg/ml). After 3 days, the wells were washed three times and fresh AIM-V medium added. Supernatants were collected from the wells after a further 7 days and IgG content determined by an ELISA.

Figure 2 shows that both IL-2 and TGF-β significantly decrease spontaneous IgG production. The values represent the mean ± SEM of IgG (μg/ml) produced by the 12 SLE patients PBMC cultured as described in legend to figure 1 except some cells were also incubated with IL-2 (10U/ml) or TGF-β (10pg/ml) only.

Figures 3A and 3B show that anti-TGF-β can reverse the effects of IL-2. SLE patients PBMC was cultured for three days in the presence (solid bars) or absence (spotted bars) of IL-2 (10U/ml). Included in these cultures was medium, anti-TGF-β (10μg/ml) or control mouse IgG1 (10μg/ml). After 3 days the wells were washed and fresh AIM-V medium added. Supernatants were collected after a further seven days and assayed for IgG (Figure 3A) or anti-NP (Figure 3B) content by an ELISA.

Figures 4A, 4B and 4C depict regulatory effects of DC8+ T cells on antibody production. (A) Synergism between NK cells and CD8+ cells in the suppression of IgG production in a healthy subject. CD4+ cells and B cells were stimulated with anti-CD2 and the effects of CD8+ cells and NK cells were examined. The combination of NK and CD8+ cells markedly inhibited anti-CD2 induced IgG production we previously reported (Gray, J.D. *et al.* (1998), *J Immunol 160:*2248-2254; Gray, J.D. *et al.* (1994), *J Exp Med 180:*1937-1942). (B) NK cells and CD8+ cells enhance IgG synthesis in SLE. CD4+ cells from a patient with active SLE and resting B cells from a healthy subject were stimulated with anti-CD2. Enhancement of IgG production by SLE CD8+ cells was markedly increased by the addition of NK cells. (C) Cytokine normalization

Circulating B lymphocytes spontaneously secreting IgG are increased in patients with active SLE (Blaese, R.M., *et al.* (1980), *Am J. Med 69:*345-350; Klinman, D.M. *et al.* (1991) *Arthritis Rheum 34:* 1404-1410). Sustained production of polyclonal IgG and autoantibodies in vitro requires T cell help (Shivakumar, S. *et al.* (1989), *J Immunol 143:*103-112). Previous studies of T cell regulation of spontaneous IgG production shows that while CD8+ T cells inhibit antibody production in healthy individuals, in SLE these cells support B cell function instead (Linker-Israeli, M. *et al.* (1990), *Arthritis Rheum 33:*1216-1225).

Accordingly, the present invention is drawn to methods of treating antibody-mediated autoimmune diseases that comprise removing peripheral blood mononuclear cells (PBMCs) from the patient with the autoimmune disease and treating the cells with an inhibitory composition.

Without being bound by theory, it appears that there are several ways the methods of the invention may work. First of all, the treatment of the cells by an inhibitory composition leads to the direct suppression of Ig production in the treated cells, which can lead to amelioration of autoimmune symptoms. Alternatively or additionally, the treatment of the cells induces regulatory cells to down regulate Ig production in other cells. Ig in this context includes all forms of Ig, including IgM, IgG, IgE, *etc.* The net result is a decrease in the amount of Ig in the system.

Thus, the present invention restores the capacity of peripheral blood T cells from patients with autoimmune disorders to down regulate antibody production by treating them with an inhibitory composition ex vivo.

Accordingly, the present invention provides methods of treating antibody-mediated autoimmune disorders in a patient. By "antibody-mediated autoimmune diseases" herein is meant a disease in which individuals develop antibodies to constituents of their own cells or tissues. Antibody-mediated autoimmune diseases include, but are not limited to, systemic lupus erythematosus (SLE), pemphigus vulgaris, myasthenia gravis, hemolytic anemia, thrombocytopenia purpura, Grave's disease, dermatomyositis and Sjogren's disease. The preferred autoimmune disease for treatment using the methods of the invention is SLE.

By "treating" an autoimmune disorder herein is meant that at least one symptom of the autoimmune disorder is ameliorated by the methods outlined herein. This may be

not done simultaneously with collection or to further purify and/or concentrate the cells. The cells may be washed, counted, and resuspended in buffer.

The PBMCs are generally concentrated for treatment, using standard techniques in the art. In a preferred embodiment, the leukophoresis collection step results a concentrated sample of PBMCs, in a sterile leukopak, that may contain reagents and/or doses of the inhibitory composition, as is more fully outlined below. Generally, an additional concentration/purification step is done, such as Ficoll-Hypaque density gradient centrifugation as is known in the art.

In a preferred embodiment, the PBMCs are then washed to remove serum proteins and soluble blood components, such as autoantibodies, inhibitors, *etc.*, using techniques well known in the art. Generally, this involves addition of physiological media or buffer, followed by centrifugation. This may be repeated as necessary. They can be resuspended in physiological media, preferably AIM-V serum free medium (Life Technologies) (since serum contains significant amounts of inhibitors) although buffers such as Hanks balanced salt solution (HBBS) or physiological buffered saline (PBS) can also be used.

Generally, the cells are then counted; in general from 1 X 10^9 to 2 X 10^9 white blood cells are collected from a 5-7 liter leukophoresis step. These cells are brought up roughly 200 mls of buffer or media.

In a preferred embodiment, the PBMCs may be enriched for one or more cell types. For example, the PBMCs may be enriched for CD8+ T cells or CD4+ T cells. This is done as is known in the art, as described in Gray *et al.* (1998), *J. Immunol. 160:*2248, hereby incorporated by reference. Generally, this is done using commercially available immunoabsorbent columns, or using research procedures (the PBMCs are added to a nylon wool column and the eluted, nonadherent cells are treated with antibodies to CD4, CD16, CD11b and CD74, followed by treatment with immunomagnetic beads, leaving a population enriched for CD8+ T cells).

Once the cells have undergone any necessary pretreatment, the cells are treated with an inhibitory composition. By "treated" herein is meant that the cells are incubated with the inhibitory composition for a time period sufficient to develop the capacity to inhibit Ig and autoantibody production, particularly when transferred back to the patient. The incubation will generally be under physiological temperature. As noted above, this may

initiate the CD2 signaling pathway. A preferred CD2 activator comprises anti CD2 antibodies (OKT11, American Type Culture Collection, Rockville MD). In general, the concentration of CD2 activator used will be sufficient to induce the production of TGF-β. The concentration of anti CD2 antibodies used ranges from about 1 ng/ml to about 10 µg/ml, , with from about 10 ng/ml to about 100 ng/ml being especially preferred.

In addition to treatment with an inhibitory composition, in some embodiments it is desirable to use a mitogen to activate the cells; that is, many resting phase cells do not contain large amounts of cytokine receptors. The use of a mitogen such as Concanavalin A can allow the stimulation of the cells to produce cytokine receptors, which in turn makes the methods of the invention more effective. When a mitogen such as ConA is used, it is generally used as is known in the art, at concentrations ranging from 1 µg/ml to about 10 µg/ml is used. In addition, it may be desirable to wash the cells with components to remove the ConA, such as α-methyl mannoside, as is known in the art.

The inhibitory composition is incubated with the cells for a period of time sufficient to cause an effect. In a preferred embodiment, treatment of the cells with the inhibitory composition is followed by immediate transplantation back into the patient. Accordingly, in a preferred embodiment, the cells are incubated with the inhibitory composition for from about 12 to about 120 hours, with from about 24 to about 72 hours being preferred, and 48 hours being particularly preferred.

In one embodiment, the cells are treated for a period of time, washed to remove the inhibitory composition, and may be reincubated. Before introduction into the patient, the cells are preferably washed as outlined herein to remove the inhibitory composition. Further incubations for testing or evaluation may also be done, ranging in time from a few hours to several days. If evaluation of Ig production prior to introduction to a patient is desirable, the cells will be incubated for several days to allow Ig production (or lack thereof) to occur.

Once the cells have been treated, they may be evaluated or tested prior to autotransplantation back into the patient. For example, a sample may be removed to do: sterility testing; gram staining, microbiological studies; LAL studies; mycoplasma studies; flow cytometry to identify cell types; functional studies, *etc.* Similarly, these and other lymphocyte studies may be done both before and after treatment.

of time, for example 3-5 times over a two week period. Generally, the amelioration of the autoimmune disease symptoms persists for some period of time, preferably at least months. Over time, the patient may experience a relapse of symptoms, at which point the treatments may be repeated.

In a preferred embodiment, the invention further provides kits for the practice of the methods of the invention, *i.e.*, the incubation of the cells with the inhibitory compositions. The kit may have a number of components. The kit comprises a cell treatment container that is adapted to receive cells from a patient with an antibody-mediated autoimmune disorder. The container should be sterile. In some embodiments, the cell treatment container is used for collection of the cells, for example it is adaptable to be hooked up to a leukophoresis machine using an inlet port. In other embodiments, a separate cell collection container may be used.

The form and composition of the cell treatment container may vary, as will be appreciated by those in the art. Generally the container may be in a number of different forms, including a flexible bag, similar to an IV bag, or a rigid container similar to a cell culture vessel. It may be configured to allow stirring. Generally, the composition of the container will be any suitable, biologically inert material, such as glass or plastic, including polypropylene, polyethylene, *etc.* The cell treatment container may have one or more inlet or outlet ports, for the introduction or removal of cells, reagents, inhibitory compositions, *etc.* For example, the container may comprise a sampling port for the removal of a fraction of the cells for analysis prior to reintroduction into the patient. Similarly, the container may comprise an exit port to allow introduction of the cells into the patient; for example, the container may comprise an adapter for attachment to an IV setup.

The kit further comprises at least one dose of an inhibitory composition. "Dose" in this context means an amount of the inhibitory composition such as cytokines, that is sufficient to cause an effect. In some cases, multiple doses may be included. In one embodiment, the dose may be added to the cell treatment container using a port; alternatively, in a preferred embodiment, the dose is already present in the cell treatment container. In a preferred embodiment, the dose is in a lyophilized form for stability, that can be reconstituted using the cell media, or other reagents.

In some embodiments, the kit may additionally comprise at least one reagent, including buffers, salts, media, proteins, drugs, *etc.* For example, mitogens can be included.

SLEDAI (Bombardier, C. *et al.* (1992), *Arthritis Rheum 35:*630-640) indices with mean values of 16.5 and 13.4 respectively.

Table 1

Profile of SLE Patients

Case	SEX	Age	Ethnicity	Duration	Medications	SLAM	SLEDAI	IgG(μ/ml)
1	F	18	AA	3yr	Nil	13	9	13.7
2	F	37	H	6mo	Nil	23	13	13.0
3	F	29	H	1yr	Nil	15	6	2.6
4	F	32	AA	4yr	Pred 5mg Ohchlor 400mg	9	6	2.5
5	F	57	A	5mo	Nil	24	19	2.2
6	F	55	H	5mo	Nil	23	22	1.5
7	F	27	H	3yr	Pred 20mg Ohchlor 400mg	13	17	1.0
8	F	21	H	2yr	Nil	18	13	1.0
9	F	36	H	15yr	Pred 20mg Ohchlor 400mg Aza 25mg	14	8	0.8
10	F	41	A	4yr	Nil	15	16	0.5
11	F	20	H	6yr	Pred 25mg	11	16	0.4
12	F	25	H	1yr	Nil	21	16	0.4

Reagents

Recombinant TGF-β and monoclonal anti-TGF-β (1D11.16) antibody, a murine IgG1, were kindly provided by Dr. Bruce Pratt (Genzyme Pharmaceuticals, Farmington, MA). Recombinant IL-10 and monoclonal anti-IL-10 (JES3-19F1) antibody, and control rat

Anti-CD2 induced IgG synthesis

The effects of CD8+ T cells cultured with or without NK cells on anti-CD2 stimulated CD4+ T cells and B cells was examined in a patient with SLE in a normal control. CD4+ and CD8+ cells were prepared from nylon non-adherent lymphocytes by negative selection using immunomagnetic beads as described previously (Gray, J.D. *et al.* (1998), *J Immunol 160:*2248-2254). For CD4+ cells the nylon non-adherent cells were stained with antibodies to CD8, CD16, CD11b and CD74. The same antibodies were used to obtain CD8+ cells except that CD4 was substituted for CD8. Purity of CD4+ cells was 95% and CD8+ cells 89%. To obtain NK cells, PBMC were added to a nylon wool column and the eluted, non-adherent cells were immediately rosetted with AET treated sheep red blood cells. The non-rosetting fraction was then stained with anti-CD3 and anti-CD74 (anti-HLA-DR) antibodies and depleted of reacting cells using immunomagnetic beads (Dynal). This resultant population contained 98% CD56+ and <0.5% CD3+ and <0.5% CD20+ lymphocytes. Since SLE B cells spontaneously secrete large amounts of IgG and because of the large amount of blood needed to prepare sufficient numbers of B cells for these studies, we substituted resting B cells from a healthy donor for patient B cells in this study. To obtain B cells, nylon wool adherent cells were immediately rosetted with SRBC to remove any T cells, and treated with 5mM L-leucine methyl ester for complete removal of monocytes and functional NK cells. The resulting population was >92% CD20+ and <0.5% CD3+.

Results

In 12 patients studied, spontaneous IgG ranged from 0.4 to 13.7 μg/ml (Fig. 1). Exposure of PBMC to IL-2 ± TGF-β for 72 hours decreased IgG synthesis in 8 of 12 cases studied by at least 50% (mean decrease 79%, p=0.008, Mann Whitney). The most dramatic decreases were observed in cases with the most marked B cell hyperactivity. The correlation between the amount of IgG secreted and percent inhibition by IL-2 and TGF-β was r = 0.647, p=0.02.

We compared the effects of IL-2 and TGF-β alone to the combination of IL-2 and TGF-β. Fig. 2 shows that each of these cytokines also inhibited IL-2 production. However, after log transformation to achieve a normal distribution of the data and applying the Bonnferoni correction for multiple comparisons, analysis of variance revealed that only the combination of IL-2 and TGF-β resulted in significant inhibition (p=0.05).

IL-10 production is increased in SLE (Llorente, L. *et al.* (1993), *Eur Cytokine Network 4:*421-427) and this cytokine can inhibit production of both IL-2 and TGF-β. In 9 cases

example shown in Fig. 3A, the addition of anti-TGF-β did not affect spontaneous IgG synthesis. Antagonism of TGF-β, however, did abolish the inhibitory effects of IL-2 on IgG synthesis. PBMC from this patient (Case C in Table 2) also spontaneously produced anti-NP antibody. Here also anti-TGF-β abolished the inhibitory effects of IL-2 on anti-NP production (Fig. 3B). In this subject, therefore, the inhibitory effects of IL-2 on spontaneous IgG and autoantibody synthesis were mediated by TGF-β. This effect of anti-TGF-β was documented in 4 of 8 cases studied. Thus, the inhibitory effects of IL-2 could either be TGF-β-dependent or independent. Examples of each effect are shown in Table 3.

Table 3

Effect of IL-2 and TGF-β on Spontaneous IgG Synthesis in SLE

Cytokines Added	Patient A: TGF-β dependent inhibition G (μgm /ml)	Patient B: TGF-β independent inhibition IgG (μgm /ml)
Medium only	2.5 (100)*	2.6 (100)
TGF-β (10 pg/ml)	1.4 (56)	2.5 (96)
IL-2 & TGF-β	0.4 (16)	0.5 (19)
IL-2 & anti- TGF-β	11.6 (464)	0.5 (19)
IL-2 & IgG1	3.6 (144)	0.6 (23)

* Percent of baseline IgG synthesis

We had the opportunity to repeat the study of on SLE patient 28 days after initiation of steroid therapy (Table 4). Before treatment spontaneous IgG synthesis was greater than 2 μg/ml of IgG. Exposure of PBMC to IL-2 markedly inhibited IgG production and TGF-β had a moderate effect. Following corticosteroid therapy, spontaneous IgG production decreased by 75%. As before, exposure of PBMC to IL-2 ± TGF-β decreased IgG production by 50%. However, this inhibition was reversed by anti-TGF-β. Here again, this effect of IL-2 could be explained by upregulation of endogenous active TGF-β.

production in SLE, especially in patients with severe disease and marked B cell hyperactivity. This study confirms previous reports indicating that IL-2 can inhibit antibody production (Hirohata, S. *et al.* (1989), *J Immunol 142:* 3104-3112 and Fast, L.D. (1992), *J Immunol 149:*1510-1515) and reveals that picomolar concentrations of TGF-β can contribute to this down-regulation. In the group of 12 patients studied, the inhibitory effect of IL-2 and TGF-β on polyclonal IgG synthesis was greater than the effect of IL-2 alone. However, the inhibitory effects of IL-2 were heterogeneous. In 4 of 8 cases studied, the inhibition was TGF-β-dependent in that a neutralizing anti-TGF-β mAb abolished the effect. In the remaining cases the down-regulatory effects of IL-2 were TGF-β-independent. Similarly, both TGF-β-dependent and independent inhibition of spontaneous anti-NP autoantibody production was documented. We also investigated the effects of antagonizing the IL-10 and adding TNF-α because of previously described abnormalities in the production of these cytokines in SLE (Llorente L. *et al.* (1993), *Eur Cytokine Network 4:*421-427; Jacob, C.O. *et al.* (1990), *Proc Natl Acad Sci 87:*1233-1237). These procedures, however, had minimal effects on spontaneous Ig synthesis where lymphocytes had been activated previously.

Others have reported that the degree of B cell hyperactivity in SLE correlates with disease activity (Blaese, R.M. *et al.* (1980), *Am J Med 69:*345-350; Klinman, D.M. *et al.* (1991), *Arthritis Rheum 34:*1404-1410). This was not the case in the present study, possibly because of concurrent drug therapy. In general, those patients with marked spontaneous Ig synthesis were untreated whereas those with less B cell activity were currently receiving prednisone. We presented one case where spontaneous IgG synthesis decreased markedly after corticosteroid therapy was begun. This patient's B cells had also been secreting anti-NP antibody before treatment, and production of this autoantibody became undetectable after steroid therapy (result not shown).

TGF-β consists of a multifunctional family of cytokines important in tissue repair, inflammation and immunoregulation (Massague, J. (1990), *Annu Rev Cell Biol 6*597-641). TGF-β is different from most other cytokines in that it is secreted as an inert precursor molecule and converted to its biologically active form extracellularly (Massague, J. (1990), *Annu Rev Cell Biol 6*597-641; Flaumenhaft, R. *et al.* (1993), *Adv Pharmacol 24:*51-76). Regulatory T cells in various experimental autoimmune models such as experimental autoimmune encephalitis (Weiner, H.L. *et al.* (1994), *Annu Rev Immunol 12:*809-837) and colitis (Neurath, M.F. *et al.* (1996), *J Exp Med*

Previously, we had investigated the regulatory effects of NK cells on antibody synthesis and reported that while the direct effect of NK cells is to up-regulate IgG synthesis (Kinter, A. *et al.* (1995), *Proc Natl Acad Sci USA 92:*10985-10989), these lymphocytes have the opposite effect when cultured with CD8+ T cells in healthy subjects (Gray, J.D. *et al.* (1994), *J Exp Med 180:*1937-1942). In SLE patients, however, the combination of CD8+ T cells and NK cells enhanced IgG production (Linker-Israeli, M. *et al.* (1990), *Arthritis Rheum 33:*1216-1225). This was again observed in the present report. While in the normal subject the addition of NK cells to CD8+ T cells markedly inhibited anti-CD2 stimulated IgG synthesis, the opposite was observed in SLE. From studies of normals we had learned that NK cell-derived TGF-β induced co-stimulated CD8+ T cells to down-regulate IgG and IgM production (Gray, J.D. *et al.* (1998), *J Immunol 160:*2248-2254). In this study IL-2 and TGF-β induced moderate suppressive activity by CD8+ T cells. It is likely, therefore, that in SLE at least one way that IL-2 and TGF-β inhibit B cell activity is by generating regulatory T cells. In addition, other lymphocyte populations treated with these or other cytokines may also down-regulate B cells activity in SLE.

Example 2

The correlation of TGF-β production to disease activity and severity

Having shown that the lymphocyte production of the total and active forms of TGF-β is decreased, we next asked whether these defects correlate with disease activity and/or severity. TGF-β1 production by blood lymphocytes from 17 prospectively studied SLE patients was compared with 10 rheumatoid arthritis (RA) patients and 23 matched healthy controls. In RA the levels of active TGF-β1 were lower than controls, but not deceased to the extent found in SLE. Levels of constitutive and anti-CD2 stimulated active TGF-β1 detected in picomolar amounts were markedly reduced in 6 untreated patients hospitalized with recent onset, very active and severe SLE and similarly reduced in 11 patients with treated, less active disease. thus, decreased production of active TGF-β1 did not correlate with disease activity. By contrast, decreased production of total TGF-β1 inversely correlated with disease activity. Thus it appears that although impaired lymphocyte secretion of the latent precursor of TGF-β1 may result as a consequence of disease activity, the ability to convert the precursor molecule to its active form may be an intrinsic cellular defect. Insufficient exposure of T cells to picomolar concentrations amounts of TGF-β1 at the time they are activated can result in impaired down-regulation of Ig synthesis.

Table 5

Clinical Characteristics of Two Groups of SLE Patients

Clinical Data	Hospitalized (n=6)	Outpatient (n=11)	p Value
Age	26.8	38.6	1.037
Sex (F/M)	6/0	9/2	
Ethnic Group (H/AA/A)	5/0/1	10/1/0	
Disease Duration (yr)	0.71	8.25	0.051
Disease Activity			
SLAM	13.3	2.9	0.014
SLEDAI	15.7	4.1	0.006
Prednisone dose (mg/day)	41.2	7.8	0.008
Active Renal disease	83%	9%	0.028
Hemolytic Anemia	67%	9%	0.064
Anti-DNA (titer)	466.7	33.0	0.064
C3	47.5	98.6	0.008
C4	13.7	18.6	0.127

Reagents

Antibodies used were supernatants of hybridomas secreting anti-CD2 (OKT11, American Type Culture Collection (ATCC), Rockville, MD, and GT2 made available by Dr. Alain Bernard, Nice, France). A monoclonal antibody recognizing TGF-β isoforms 1,2 &3 (1D11), an antibody against TGF-β isoforms 2&3 (3C7), and rTGF-β2 were kindly provided by Dr. Bruce Pratt (Genzyme Pharmaceuticals, Farmington, MA).

Isolation of blood lymphocytes

Peripheral blood mononuclear cells (PBMC) were prepared from heparinized venous blood by Ficoll-Hypaque (Pharmacia, Piscataway, NJ) density gradient centrifugation using methods described previously (Ohtsuka, K. *et al.* (1998), *J Immunol 160:*2539-2545). The mononuclear cells were washed in PBS with 5mM EDTA (Life Technologies, Grand Island, NY) to remove platelets, which are a rich source of TGF-β. Peripheral blood lymphocytes (PBL) were separated from PBMC by centrifugation through a continuous Percoll (Pharmacia) density gradient. The

We measured constitutive and stimulated TGF-β1 produced by PBL from patients with SLE or RA, and compared these values with those from normal controls. The cytokine detected in culture supernatants was neutralized by a mAb recognizing isoforms 1,2,&3, but not by one against isoforms 2&3, a result confirming the production of TGF-β1. Compared to normal controls, constitutive production of active TGF-β1 was significantly decreased in SLE (14 ±5 vs 56 ±21 pg/ml, p=0.02, Fig. 5). Anti-CD2 stimulated active TGF-β1 was also decreased (87 ±22 vs 399 ±103 pg/ml, p=0.003). In RA, the mean value for constitutive TGF-β1 was similar to that of SLE (19 ±5 pg/ml) and after stimulation by anti-CD2 was intermediate between normal and SLE (197 ±54 pg/ml).

Constitutive total TGF-β1 produced by lymphocytes was also decreased in SLE in comparison with the normal group (286 ±82 vs 631 ±185 pg/ml, p=0.05). The value in RA was intermediate between normal and SLE (435 ±161 pg/ml). Following the addition of anti-CD2, total TGF-β1 increased in SLE somewhat more than in normal controls so that the differences were not statistically significant. Values in the RA group were again intermediate between the normal and SLE group.

To look for a possible relationship between decreased levels of TGF-β1 and disease activity, we compared hospitalized SLE patients with those seen in the outpatient clinic. The clinical characteristics of these two groups are summarized in Table 5. Those that were hospitalized were younger; 5 of 6 had symptoms for less than 3 months; they had markedly active disease; and most had severe SLE with nephritis and/or hemolytic anemia. The outpatient group by contrast, had chronic disease which had become less active following treatment. Notwithstanding this marked difference in disease heterogeneity, duration, activity, and severity, both constitutive and stimulated active TGF-β1 production were significantly decreased in both groups in comparison with normal controls (Table 6).

strongly with the SLEDAI index, which is weighted for major organ system involvement, also suggests a relationship with disease severity.

This study also included a control group of RA patients whose disease activity was comparable to SLE patients with established disease. Although TGF-β1 values in the RA group was somewhat less than the normal controls, with the exception of constitutive active TGF-β1, the magnitude of the defect was not as marked as in SLE and was not statistically significant.

Previously, we have documented that NK cells are the principal lymphocyte source of TGF-ß and the only lymphocyte population to constitutively produce this cytokine in its active form (Gray, J.D. *et al.* (1998), *J Immunol 160:*2248-2254). It was of interest, therefore, to find that constitutive production of NK cell-derived TGF-ß was decreased in SLE. We also learned that both IL-2 and TNF-α could enhance the production of active TGF-ß. Production of both of these cytokines are decreased in SLE (Gray, J.D. *et al.* (1994), *J Exp Med 180:*1937-1942). However, in most patients exogenous IL-2 and TNF-α could not restore TGF-ß production to normal (Example 2). IL-10 production is increased in SLE (Llorente, L. *et al.* (1993), *Eur Cytokine Network 4:*421)) and correlations between elevated levels and disease activity have been reported (Housslau, F.A. *et al.* (1995), *Lupus 4:*393-395; Haglwara, E. *et al.* (1996), *Arthritis Rheum 39:*379). IL-10 can inhibit IL-2, TNF-α and TGF-β production (Example 2 and Moore, K.W. *et al.* (1993), *Ann Rev Immunol 11:*165-190). The findings that production of active TGF-β is decreased in patients with mild as well as active disease, and that we could only partially reverse the production defect by antagonizing IL-10 (Example 2), suggests that increased IL-10 production, by itself, cannot account for decreased lymphocyte production of active TGF-ß1 in SLE. Several mechanisms are probably involved. It is likely that one or more defects in the extracellular conversion of the latent precursor to the mature, active form may explain this abnormality.

Although TGF-ß has well documented inhibitory properties on lymphocyte proliferation and effector cell function (Letterio, J.J. *et al.* (1998), *Ann Rev Immunol 16:*137-162), stimulatory properties have also been reported (Lee, H.M. *et al.* (1991), *J Immunol 151:*668-677). TGF-ß modulates cytokine production by stimulated T cells as well as up-regulating its production. In mice, TGF-ß1 selectively activates $CD8^+$ T cells to proliferate (Lee, H.M. *et al.* (1991), *J Immunol 151:*668-677), and augments the maturation of naive cells to memory T cells (Lee,

Example 4

Treating cells with a mixture of cytokines and mitogens

In this example, IgG production is down regulated by treating the cells with an inhibitory composition comprising a mixture of cytokine and mitogen. The cells are prepared as outlined in the above examples, and then they are incubated with the mixture to augment the population of cells that down regulate antibody production, such as physiological concentrations of Con A, IL-2 and TGFβ, or Con A and IL-2, for 4 to 72 hours using standard incubation techniques.

After the cells have been incubated with the cytokines and mitogen, the cells are then washed with HBBS to remove any cytokine and mitogen that are in the solution. The cells are then suspended in 200-500 ml of HBBS and are reintroduced into the mammal.

11. A kit according to claim 7 wherein said cell treatment container further comprises at least one reagent.

12. A kit according to claim 7 wherein said cell treatment container further comprises a sampling port to enable the removal of a fraction of said cells for analysis.

13. A kit according to claim 7 further comprising an exit port adapted to enable transport at least a portion of said cells to said patient.

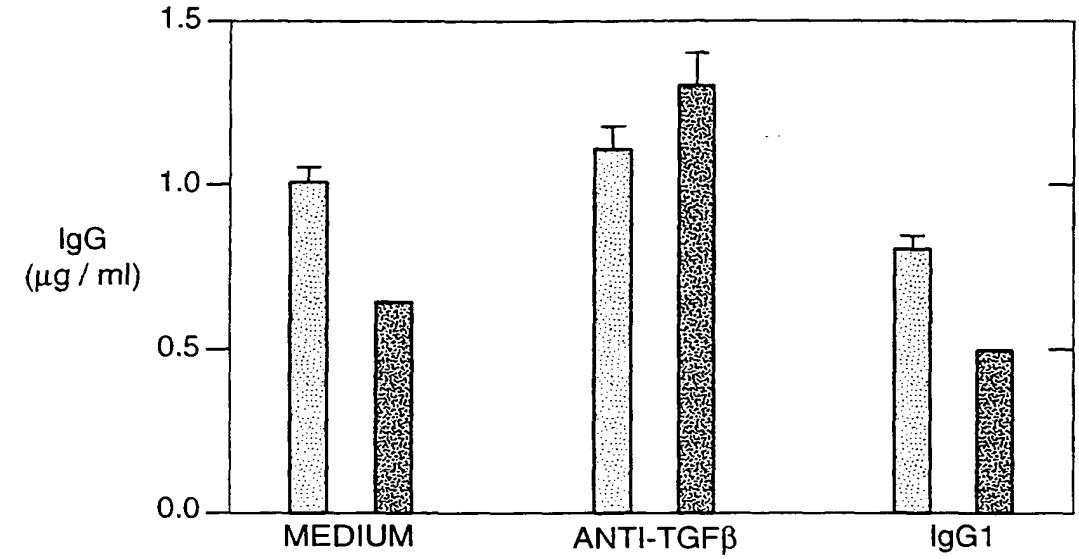
1.5
1.0
0.5
0.0
IgG
(μg / ml)
MEDIUM
ANTI-TGFβ
IgG1

FIG._3A

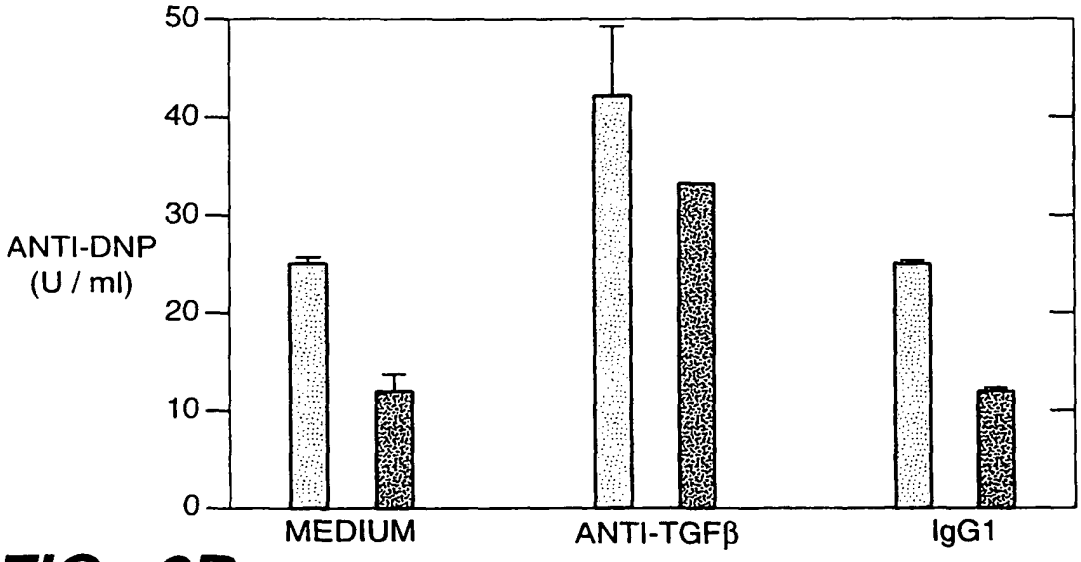
50
40
30
20
10
0
ANTI-DNP
(U / ml)
MEDIUM
ANTI-TGFβ
IgG1

FIG._3B

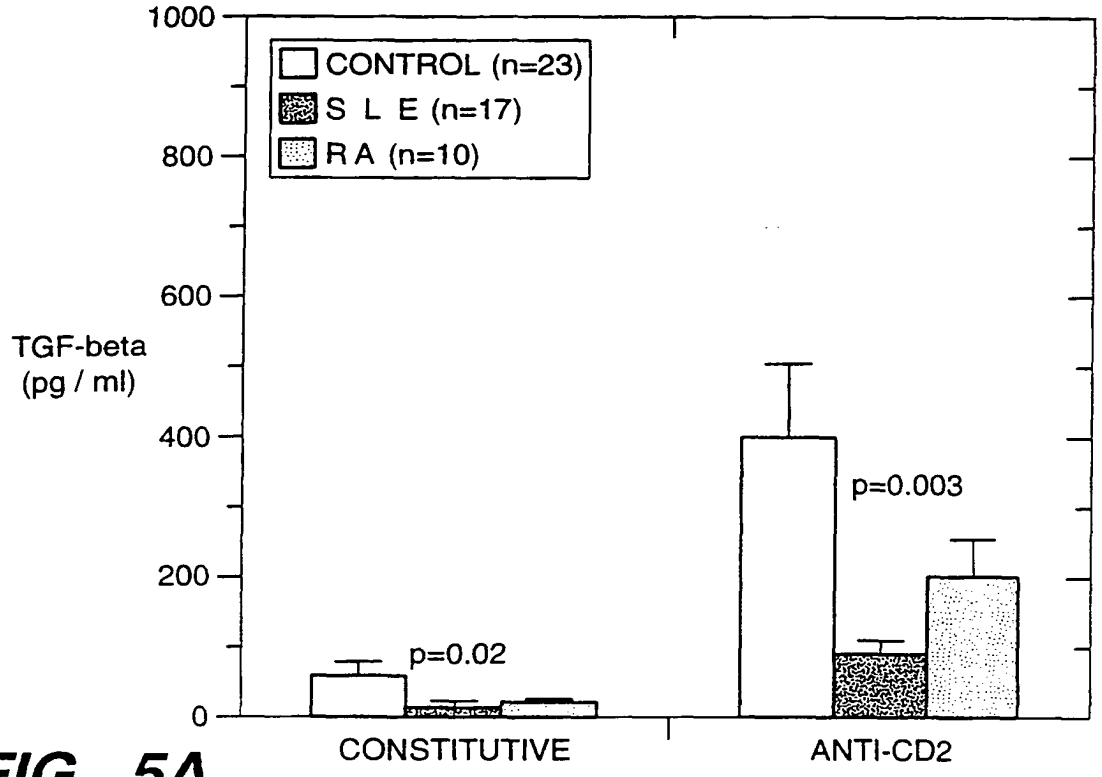
1000
800
600
400
200
0
TGF-beta
(pg / ml)
CONTROL (n=23)
S L E (n=17)
R A (n=10)
p=0.02
p=0.003
CONSTITUTIVE
ANTI-CD2

FIG._5A

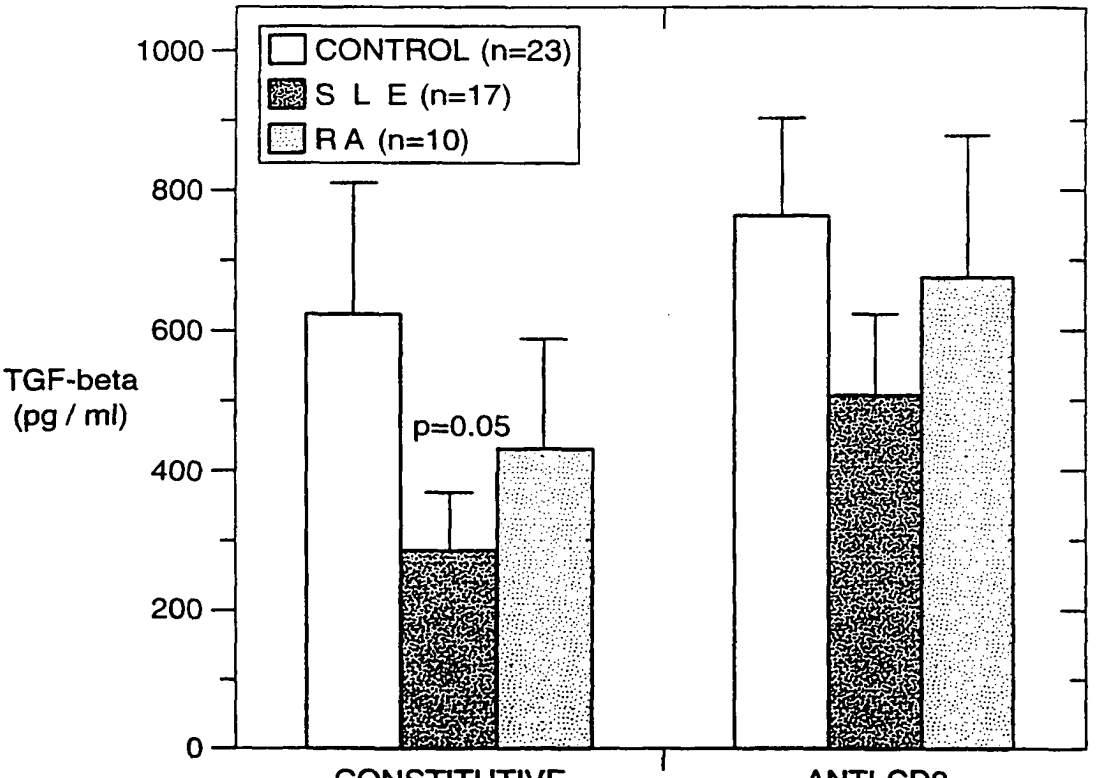
1000
800
600
400
200
0
TGF-beta
(pg / ml)
CONTROL (n=23)
S L E (n=17)
R A (n=10)
p=0.05
CONSTITUTIVE
ANTI-CD2

FIG._5B

INTERNATIONAL SEARCH REPORT

Inter. ..onal Application No
PCT/US 98/23584

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
P,X	GRAY J D ET AL: "Generation of an inhibitory circuit involving CD8+ T cells, IL-2, and NK cell-derived TGF-beta: contrasting effects of anti-CD2 and anti-CD3." JOURNAL OF IMMUNOLOGY, (1998 MAR 1) 160 (5) 2248-54, XP002099413 cited in the application see the whole document ---	1-13
P,X	OHTSUKA K ET AL: "Decreased production of TGF-beta by lymphocytes from patients with systemic lupus erythematosus." JOURNAL OF IMMUNOLOGY, (1998 MAR 1) 160 (5) 2539-45, XP002099414 cited in the application see the whole document -----	1-13

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